1,2-Diacylglycerol Content in Thoracic Aorta of Spontaneously Hypertensive Rats

Kenji Okumura, Junichiro Kondo, Yoshihisa Shirai, Masahito Muramatsu, Yumiko Yamada, Hidekazu Hashimoto, and Takayuki Ito

Phosphoinositide metabolism participates in the control of cell calcium homeostasis. Because a notable neutral lipid (1,2-diacylglycerol) is generated from phosphoinositide hydrolysis and is assumed to be a secondary messenger, we determined 1,2-diacylglycerol content and its fatty acid profiles in the thoracic aorta of spontaneously hypertensive rats (SHR) and compared it with those of normotensive Wistar-Kyoto (WKY) rats. After the aorta was exposed to $10^{-5} \text{M}$ norepinephrine as a stimulant, 1,2-diacylglycerol content in SHR was significantly higher by 33% than in WKY rats at 4 weeks of age, whereas there was no difference in 1,2-diacylglycerol content between the two strains at 20 weeks of age. Before norepinephrine stimulation, there was no significant difference in 1,2-diacylglycerol level between the two strains at 4 weeks of age. Analysis on a gas chromatograph showed that 1,2-diacylglycerol was composed of similar molecular species of fatty acids in aortas obtained from SHR and WKY rats. On the other hand, the cholesterol content of aortas was higher in SHR than in WKY rats at 20 weeks of age, whereas the difference at 4 weeks was not significant. Phosphatidylcholine, phosphatidylethanolamine, and triglyceride showed no significant difference between the two strains. It is concluded that norepinephrine-induced 1,2-diacylglycerol production increases in the thoracic aorta of SHR before the development of hypertension.

Hypertension 1990;16:43-48

The cellular mechanisms that cause genetically determined elevations of blood pressure in spontaneously hypertensive rats (SHR) have not been completely defined, although an increase in peripheral resistance results from increased active tension in the vascular smooth muscle. This is likely to be a reflection of an increased concentration of free calcium in vascular smooth muscle cells. However, it has not been elucidated why abnormalities of calcium handling occur in the development of genetic hypertension in SHR as well as humans.

Recently, it has become clear that phosphoinositide metabolism evoked by hormones is involved in intracellular Ca$^{2+}$ mobilization. In this system, phosphoinositiode hydrolysis produces two intracellular second messengers: inositol trisphosphate, which is known to induce Ca$^{2+}$ release from the stores, and 1,2-diacylglycerol (DAG), which causes the activation of protein kinase C. We estimated the mass measurement of 1,2-DAG in heart tissues by thin-layer chromatography (TLC)/flame ionization detector (FID) in previous studies. However, an accurate determination of 1,2-DAG in a lipid extract from blood vessels could not be achieved with this method because of the difficulty in separating a large amount of triglyceride from the internal standard. Therefore, we recently established a method for 1,2-DAG quantitation in blood vessels using a stepwise elution procedure and TLC/FID. In the present study, we determined 1,2-DAG content and its fatty acid composition in the aorta from SHR and normotensive Wistar-Kyoto (WKY) rats. In addition, cholesterol and other lipid contents in the aorta were evaluated.

Methods

Animals

Male SHR and WKY rats were obtained from Charles River Japan, Atsugi, Japan at least 1 week before the experiment and were fed a standard pellet diet and water ad libitum. Indirect systolic blood pressure measurements using tail-cuff plethysmography were taken on all rats to ensure that each rat had been correctly classified as either hypertensive or normotensive. Food was withdrawn 8 hours before the rats were killed by decapitation, but they were given free access to water.

Tissue Preparation

The descending thoracic aorta was removed and washed with ice-cold Krebs-Henseleit buffer of the
following composition (mM): NaCl 120, NaHCO₃ 25, KCl 4.8, KH₂PO₄ 1.2, MgSO₄ 1.25, CaCl₂ 1.25, and glucose 8.6 (pH 7.4). Adipose tissue and blood clots were carefully trimmed. The thoracic aorta was preincubated in Krebs-Henseleit buffer gassed continuously with 95% O₂ and 5% CO₂ and maintained at 37°C. After preincubation for a 20-minute stabilization period, the aorta was exposed to 10⁻⁵ M l-norepinephrine bitartrate (Sigma Chemical Co., St. Louis, Mo.) in Krebs-Henseleit solution up to a period of 10 minutes. Then, tissue samples were immediately frozen in liquid N₂.

Extraction and Analysis of Lipids

Crude lipids were extracted according to Folch et al. with some modification. The sample was placed in 5.5 ml ice-cold chloroform/methanol mixture (2:1, vol/vol) containing 0.01% butylated hydroxytoluene as an antioxidant and cholesteryl acetate (0.03 mg/ml), an internal standard, and homogenized for 20 seconds in a motor-driven glass-glass homogenizer kept in ice. The homogenized tissue was chilled on ice for 30 minutes and then filtered through Toyo filter paper. For the determinations of triglyceride and phospholipids, 0.5 ml of the lipid extract was transferred into another tube. The remaining filtrate was evaporated to dryness under a stream of N₂ at 40°C. Dried lipids were resuspended in 0.5 ml n-hexane/diethyl ether (98:2, vol/vol) and were applied to a 0.5 ml silicic acid (minus 325 mesh from Bio-Rad, Richmond, Calif.) column (20 mm x 5.5 mm i.d.) equilibrated with n-hexane. Four sequential elutions were obtained by stepwise elution according to a method described recently. After the second fraction (n-hexane/diethyl ether, 95:5, vol/vol) was discarded to eliminate most of the triglyceride in the extract, the first fraction (n-hexane/diethyl ether, 98:2, vol/vol) containing an internal standard and the third elution (diethyl ether) containing cholesterol and 1,2-DAG were combined. The eluates were concentrated under a stream of N₂ at 40°C and dissolved in 20 μl chloroform. One to two microliters of the eluted lipids was applied carefully to the Chromarods (Iatron Laboratories, Tokyo). Two solvent systems were used. The first and second developments were carried out in a solvent system of n-hexane/diethyl ether/chloroform/acetic acid (46:6:0.05, vol/vol/vol/vol) until the solvent front had migrated approximately 8 cm. The third development was carried out in a solvent system of n-hexane/diethyl ether/acetic acid (98:1:1, vol/vol/vol) in the same direction until the solvent front had migrated approximately 11 cm. Chromarods were then dried at 50°C and scanned with an Iatroscan TH-10 (Iatron). Triglyceride and phospholipids were also quantitated by the TLC/FID system.

Fatty Acid Composition of 1,2-Diacylglycerol

After the quantification of individual lipids using the Iatroscan methods, the residual neutral lipids were separated by TLC on precoated silica gel plates (20 x 20 cm, Kieselgel 60 F₂₅₄ from Merck, Darmstadt, FRG). The plates were subjected to development twice in a solvent system containing n-hexane/diethyl ether/acetic acid (80:35:1, vol/vol/vol). The spot corresponding to 1,2-DAG was identified by 1,2-diolein (Sigma), scraped off, and eluted with 1.5 ml chloroform/methanol (9:1, vol/vol). After the extract was filtered through Whatman GF/C glass fiber filters and evaporated to dryness with N₂ gas, fatty acids in this fraction were transmethylated with a boron trifluoride (BF₃)-methanol according to the method of Morrison and Smith. The tube containing the dried 1,2-DAG and 0.3 ml of a solution of 14% BF₃ in methanol (Wako Chemical Co., Osaka, Japan) was heated in a boiling water bath for 10 minutes. The methyl esters of fatty acids were extracted by the addition of 0.2 ml water and 0.5 ml n-hexane and mixed vigorously for 1 minute. The upper layer was transferred and concentrated under a stream of N₂ gas. The fatty acid composition of 1,2-DAG was analyzed on a gas chromatograph (GC 14-A model apparatus, Shimadzu Co., Kyoto, Japan) equipped with an FID and a fused silica capillary column of HR 20M (25 m x 0.25 mm i.d., Shinwakagaku, Kyoto, Japan). Peaks were identified by comparison with standards (Nu-Chek-Prep Inc., Elysian, Minn.), and the relative percentages of peak areas were calculated by a Chromatopac C-R5A integrator (Shimadzu).

Statistics

The results are expressed as the mean±SEM, and comparisons between the two different groups were assessed with a two-way analysis of variance and Duncan's test except for the fatty acid composition of 1,2-DAG, where the Mann Whitney U test was used. A p value of less than 0.05 was considered statistically significant.

Results

There was no difference in the systolic blood pressure at 4 weeks of age between SHR and WKY rats, whereas the systolic blood pressure of 20-week-old SHR was significantly higher than that of the age-matched WKY rats as shown in Table 1. The triglyceride contents in aortas of SHR were apparently higher than in WKY rats at 4 and 20 weeks of age; the differences, however, did not reach statistical significance. No significant difference was found in phosphatidylethanolamine or phosphatidylcholine between the two strains (Table 1). Only major phospholipids were evaluated in this study because minor species of phospholipids were not completely separated from other small peaks.

The relations between weight ratio and peak area ratio through the entire extraction and assay procedure of 1,2-DAG (1,2-diolein) and cholesterol compared with cholesteryl acetate are illustrated in Figure 1. They reveal linearity throughout the ratio range tested.

After exposure to 10⁻⁵ M norepinephrine for 10 minutes, 1,2-DAG content in the descending thoracic
TABLE 1. Systolic Blood Pressure and Myocardial Lipid Contents in Thoracic Aorta

<table>
<thead>
<tr>
<th>Variable</th>
<th>4 weeks WKY</th>
<th>4 weeks SHR</th>
<th>20 weeks WKY</th>
<th>20 weeks SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>92±2</td>
<td>94±1</td>
<td>147±1</td>
<td>184±1*</td>
</tr>
<tr>
<td>Triglyceride (μg/mg wet wt)</td>
<td>49.4±8.6</td>
<td>64.5±8.7</td>
<td>31.9±7.1</td>
<td>57.0±15.3</td>
</tr>
<tr>
<td>Phosphatidylethanolamine (μg/mg wet wt)</td>
<td>1.79±0.14</td>
<td>1.82±0.13</td>
<td>1.44±0.27</td>
<td>1.60±0.19</td>
</tr>
<tr>
<td>Phosphatidylcholine (μg/mg wet wt)</td>
<td>2.65±0.21</td>
<td>2.57±0.19</td>
<td>2.16±0.25</td>
<td>2.08±0.09</td>
</tr>
</tbody>
</table>

Results represent mean±SEM obtained from eight rats. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.

*aIndicates p<0.01 compared with age-matched WKY rats.

Aortas from 4-week-old SHR averaged 77.5±4.8 ng/mg wet wt, which was significantly higher by 33% than in those of WKY rats (58.2±5.2 ng/mg wet wt, p<0.05) (Figure 2). On the other hand, there was no difference in 1,2-DAG contents between 20-week-old WKY rats and SHR (68.6±8.0 and 69.3±8.3 ng/mg wet wt, respectively). 1,2-DAG in the aortas was enriched in 16:0, 16:1, 18:0, and 18:1 in both SHR and WKY rats. The unexpressed 1,2-DAG components were 20:3, 22:1, and unidentified peaks. The profile of 1,2-DAG fatty acids revealed no significant difference between the two strains at 4 or 20 weeks of age (data at 4 weeks is shown in Table 2).

In contrast to 1,2-DAG, a significant difference in the cholesterol contents of the thoracic aortas was observed at 20 weeks of age. Cholesterol contents of the aortas were higher in 20-week-old SHR than in age-matched WKY rats (0.91±0.05 and 0.72±0.04 μg/mg wet wt, respectively; p<0.01) (Figure 3). In both strains, cholesterol contents were higher at the age of 4 weeks than at 20 weeks (p<0.01).

Figure 4 shows a time course of 1,2-DAG content of 4-week-old rats. There was no significant difference in 1,2-DAG in the unstimulated aortas between WKY rats (42.6±2.4 ng/mg wet wt) and SHR (44.0±2.6 ng/mg wet wt) after a preincubation period of 20 minutes. After exposure to 10⁻⁵ M norepinephrine, 1,2-DAG accumulation in the aortas from SHR was induced more than in WKY rats.

Discussion

These results demonstrate that 1,2-DAG contents of the aortas after stimulation by norepinephrine, one of the most important chemical mediators, were enhanced in 4-week-old SHR before the development of established hypertension when compared with age-matched WKY rats but that this phenomenon disappeared at 20 weeks of age. It is important to evaluate 1,2-DAG in stimulated aortas in the bath because the incubation without any stimulation appears to be unrepresentative of physiological conditions, and 1,2-DAG content in the aortas after excision may lead to mistaken conclusions because of intervention accompanied by the excision. We have recently shown that the norepinephrine-stimulated aortas showed no significant change in cholesterol content, although the 1,2-DAG content increased nearly twofold after thoracic aortas were exposed to 10⁻⁵ M norepinephrine for a period of 10 minutes.7
The short duration of norepinephrine stimulation also did not affect phospholipid species or cholesterol contents in the myocardium despite an enhancement in 1,2-DAG production.\(^1\)

Recently, several studies have reported defective phosphoinositide metabolisms in genetically developed hypertension (reviewed in References 11 and 12). In platelets, thrombin-stimulated phospholipase C activity, which cleaves the lipid to yield 1,2-DAG, is enhanced in SHR.\(^1\) Moreover, the finding that enhanced protein kinase C activity has been observed in SHR platelets\(^13\) is consistent with increased phosphoinositide metabolism in SHR platelets. Also, studies of erythrocytes in SHR showed an increase in phospholipase C activity and a decrease in DAG kinase activity resulting in DAG accumulation in erythrocytes.\(^14\) With respect to aortas obtained from SHR, abnormalities in phosphoinositide turnover have also been reported. Phospholipase C activity in the descending aorta was enhanced in both 4- and 14-week-old SHR when compared with that of age-matched WKY rats.\(^15\) Furthermore, a high dose of vasopressin resulted in higher concentrations of intracellular calcium in cultured vascular smooth muscle cells from SHR than from WKY rats.\(^2\) The production of labeled inositol phosphates was significantly enhanced in the aortas of 5-week-old SHR, either unstimulated or in the presence of \(10^{-4}\) M norepinephrine, whereas hydrolysis of inositol phospholipids on exposure to norepinephrine was conversely lower at 19 weeks in SHR than in WKY rats.\(^16\) No difference in 1,2-DAG contents in established hypertension herein implies the possibility that there exists a defect in coupling between receptors and the subsequent functions in SHR with aging.\(^17,18\)

An accumulation of 1,2-DAG in the tissue in response to stimulants is believed to be responsible for the activation of protein kinase C. Protein kinase C has multiple substrates and is a regulatory element in a variety of cellular functions, in particular, cellular proliferation and differentiation by the expression of \(c\)-myc messenger RNA.\(^19,20\) There is much evidence suggesting that protein kinase C plays an important role in maintaining isometric forces in vascular smooth muscle.\(^21,22\) 1,2-DAG formation, which is sustained in contrast to inositol trisphosphate\(^23\) (a concomitant product from phosphoinositide hydrolysis), is suggested to produce slow developing and sustained constriction in various smooth muscle preparations like phorbol esters.\(^24,25\) It has been reported that the determination of protein kinase C activity in soluble and particulate fractions from 20- to 23-week-old SHR aortas did not reveal significant differences compared with those of WKY rats.\(^26\) These results do not conflict with those of 1,2-DAG demonstrated here at 20 weeks.

It has been suggested that catecholamines\(^7\) and angiotensin II\(^23\) stimulate 1,2-DAG production in the blood vessels resulting from phosphoinositide hydrolysis. Accordingly, an increase in 1,2-DAG accumulation after incubation in the presence of norepinephrine

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**TABLE 2. Fatty Acid Composition of 1,2-Diacylglycerol in Thoracic Aorta From 4-Week-Old Rats**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>WKY</th>
<th>SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>8.00±0.31</td>
<td>7.72±0.24</td>
</tr>
<tr>
<td>16:0</td>
<td>25.34±0.76</td>
<td>25.50±0.44</td>
</tr>
<tr>
<td>16:1 isomers</td>
<td>12.92±0.95</td>
<td>14.03±0.71</td>
</tr>
<tr>
<td>18:0</td>
<td>14.13±0.65</td>
<td>13.67±0.74</td>
</tr>
<tr>
<td>18:1 isomers</td>
<td>13.58±0.27</td>
<td>13.10±0.42</td>
</tr>
<tr>
<td>18:2 isomers</td>
<td>6.60±0.47</td>
<td>6.13±0.18</td>
</tr>
<tr>
<td>20:1</td>
<td>2.60±0.52</td>
<td>3.31±0.33</td>
</tr>
<tr>
<td>20:4</td>
<td>(n=6) 3.77±0.16</td>
<td>4.08±0.22</td>
</tr>
<tr>
<td>22:5</td>
<td>(n=9) 1.60±0.16</td>
<td>1.74±0.21</td>
</tr>
<tr>
<td>22:6</td>
<td>(n=3) 1.60±0.13</td>
<td>1.63±0.18</td>
</tr>
</tbody>
</table>

Results represent mean±SEM obtained from eight rats. Fatty acids are expressed as percent area of chromatograms. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats. All differences are not significant (\(p>0.05\)).
is thought to be due to enhanced phosphoinositide hydrolysis. Unfortunately, this study did not attempt to demonstrate evidence of 1,2-DAG production through phosphoinositide breakdown or inositol trisphosphate production. 1,2-DAG is also suggested to be generated from other compounds such as phosphatidylcholine, 27, 28 phosphatidic acid, 29 and glycolipids 30 in other tissues. These alternative productions are considered to be one reason why elevated 1,2-DAG concentration is sustained on stimulation. 29

The fatty acid moieties involved in 1,2-DAG in tissues may not be equivalent activators of protein kinase C, as the exogenous addition of 1,2-DAG and its analogues have different biological potentials for activating protein kinase C. 31, 32 However, few studies of the molecular species of 1,2-DAG relying on chemical mass data have been performed, although those using labeled fatty acids or glycerol have been conducted. Our findings show that 1,2-DAG was composed of similar molecular species of fatty acids in SHR and WKY rats at both 4 and 20 weeks of age, indicating that the subclass of 1,2-DAG production is distributed equally. 1,2-DAG is conceived to be a potential source of arachidonic acid, a precursor of prostaglandins and prostacyclin. However, the arachidonic acid content in 1,2-DAG was modest in this study as previously described in the pancreas 33 and in the myocardium. 34 At present, the definite involvement of arachidonic acid in 1,2-DAG remains to be explored.

Cholesterol plays an important structural role in the lipid core of biological membranes. The high concentrations of cholesterol have an influence on membrane lipid core of biological membranes. The high concentration is sustained on stimulation. 29

References
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Key Words • diacylglycerol • cholesterol • norepinephrine • aorta • spontaneously hypertensive rats
1,2-diacylglycerol content in thoracic aorta of spontaneously hypertensive rats.
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Hypertension. 1990;16:43-48
doi: 10.1161/01.HYP.16.1.43

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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